Opsin Structure Probed by Raman Spectroscopy of Photoreceptor Membranes

Abstract. *The first nonresonance Raman spectra of photoreceptor membranes are presented. Information about the membrane protein, opsin, and the membrane phospholipids can be deduced. Opsin appears to contain α-helical structure but little β structure. The tyrosine residues are predominantly hydrogen bonded, and disulfide bonds, if they are present, are not in the normal* gauche-gauche *configuration.*

Elucidating the structure and function of photoreceptor membranes remains a key goal in vision research (*1–3*). In vertebrate rod cells, these membranes are found in the form of 200-Å-thick disks which are stacked inside the rod outer segment. Much interest has focused on rhodopsin, the major protein component of disk membrane, since its interaction with light is the first basic event in dim light vision, an event which may be directly coupled to permeability changes in the disk membrane (*4*). However, the fact that the membrane protein rhodopsin is not soluble in most solvents has made crystallization thus far impossible, and has presented formidable difficulties in applying standard biochemical approaches.

The demonstration that Raman spectroscopy can provide useful information about the conformation of biological molecules (*5–7*) has led investigators to attempt to use this technique to obtain new information on intact biological membranes (*8, 9*). The large number of proteins in these membranes has precluded the possibility of deducing specific information about individual membrane proteins. Opsin makes up 80 percent of the total protein complement in the disk membrane (*1–3*) and is thus amenable to investigation by this technique.

We report here the first nonresonance Raman spectroscopic study of the in situ components of the photoreceptor membrane. Resonance-enhanced Raman spectra (*6, 7*), which are sensitive primarily to the retinylidene chromophore, have been reported from both cattle rhodopsin extracted into digitonin micelles (*10*) and intact bovine photoreceptor membranes (*6, 11*). In contrast, nonresonance Raman spectra of photoreceptor membranes are difficult to obtain because of the presence of the highly absorbing retinylidene chromophore. Because of its low solubility in aqueous media, retinal, even after bleaching, cannot be easily extracted from the membrane without disrupting the structure (*12*). It remains in the membrane phase either free (absorption maximum, λ_{max}, $\simeq$ 380 nm) or randomly bound as an unprotonated (λ_{max}, $\simeq$ 365 nm) or protonated (λ_{max}, $\simeq$ 450 nm) Schiff's base and will completely dominate the Raman spectrum unless the laser exciting wavelength is tuned far from the chromophore absorption (for example, into the infrared).

One approach to this problem is to reduce the chromophore enzymatically to retinol by bleaching in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which activates the retinal oxidoreductase, another photoreceptor membrane protein (*13*). The opsin membrane that results can then be isolated from the retinol, and high quality Raman spectra are obtained. In contrast to resonance Raman studies, Raman spectroscopy of opsin membranes reveals details about the conformation of the protein opsin, the end product of physiological bleaching, as well as the membrane phos-

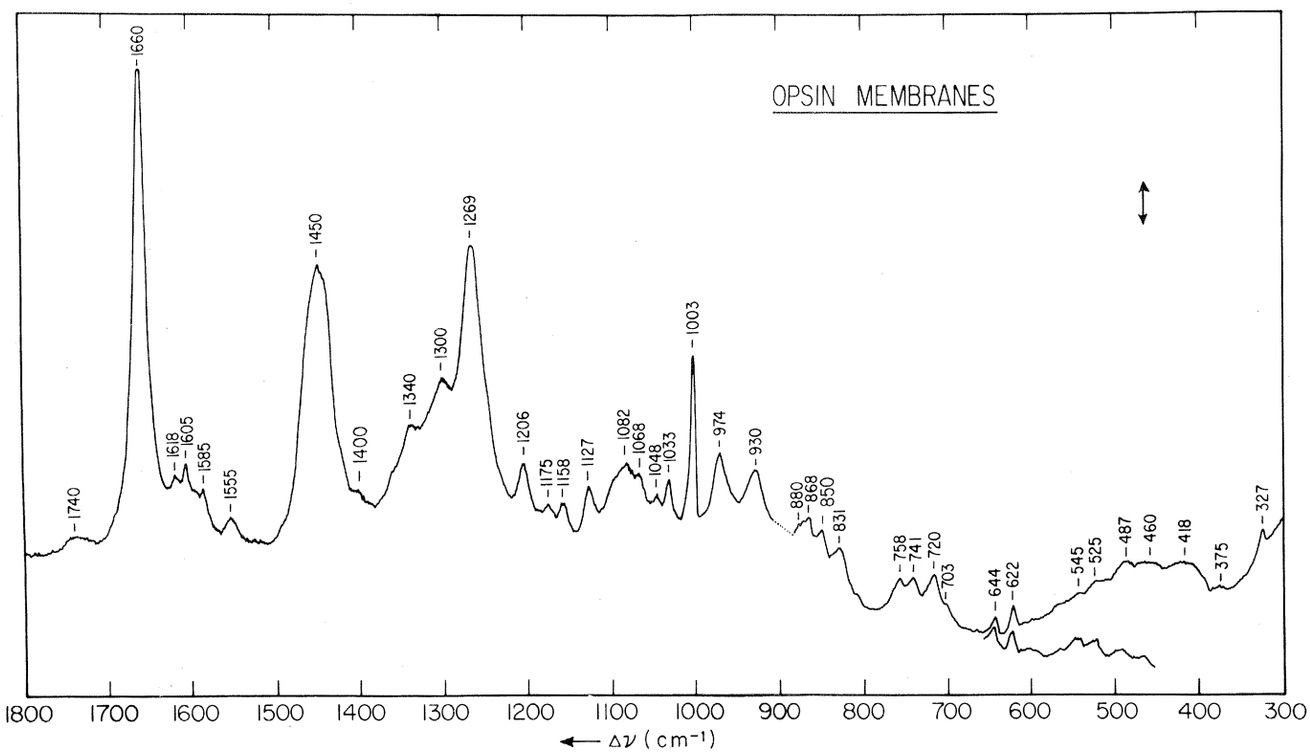

Fig. 1. Typical Raman spectrum of calf opsin membranes, recorded at 24°C. The pellet sample is held in a Kimax melting-point glass capillary (1.6 mm diameter) that is in a temperature-controlled sample holder. Spectra were measured with a SPEX Ramalog IV system and the 514.5-nm line of an Ar^+ laser (Spectra-Physics 164-03), with incident power 75 mw. Scanning speed was 3 cm^{-1}/min, slit width 200 μm, averaging time 100 seconds, and the vertical arrow represents 100 count/sec. Other aspects of the system have been described (*6, 7*). Spectra were repeated from different membrane preparations, with different laser lines (457.9 and 488.0 nm) and various temperatures. Inset shows 450 to 650 cm^{-1} region recorded with less fluorescent background. A spurious grating ghost was found near 900 cm^{-1} (dotted line). The opsin membranes are estimated to contain 50 percent lipid and 40 percent protein by weight (*2, 3*).

pholipids. Circular dichroism (CD) studies of rhodopsin and opsin membranes indicate a conformational equivalence as long as phospholipid remains associated (*14*).

Retinas were excised from calf eyes within 2 hours of removal from the animals. In order to reduce retinal to retinol, the retinas were bleached for 1 hour under an unfiltered 100-watt incandescent lamp in the presence of 5 m*M* NADPH and 1 m*M* dithioerythritol (DTE) (Sigma). Opsin was isolated by a procedure based on the sucrose gradient method (*3*). An isotonic aqueous buffer consisting of 15 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, Sigma), *p*H 7.1, 3 m*M* $MgCl_2$, 10 m*M* KCl, and 140 m*M* NaCl was used in the homogenization of the retinas and in washing and storing the opsin membranes. This treatment appears to leave the rod outer segments unlysed. In some cases, a D_2O buffer of the same composition as above was substituted during the washing. Then 3 m*M* *n*-propyl gallate (Sigma), an antioxidant, and 1 m*M* DTE, which protects sulfhydryl groups, were added to the sucrose gradients. Under these conditions disulfide bonds (cystine residues) remain unaffected. Absorption measurements of rhodopsin isolated in parallel to opsin, quantitative regeneration of the opsin with the use of 11-*cis*-retinal, sodium dodecyl sulfate (SDS), polyacrylamide gel electrophoresis, and protein determination by a modified Lowry procedure (*15*) were used as controls for purity and regenerability of the membranes (*12*). A highly predominant band with a molecular weight of 37,000 was found by polyacrylamide gel electrophoresis, in agreement with the estimated molecular weight of opsin (*2, 3*). The other controls indicated that the opsin membranes could be regenerated and were of high purity (*12*).

As shown in Fig. 1, Raman spectra (with little noise) of opsin membranes can be obtained which compare favorably with spectra reported for simpler biological materials (*5–7, 16–18*). Spectra recorded from opsin membranes prepared at different times are virtually identical. Spectra recorded at room temperature and 5°C also did not differ, except in the 1062 and 1082 cm^{-1} phospholipid vibrations.

Most of the peaks shown in Fig. 1 can be associated with protein and lipid group vibrations (Table 1). Assignments were made by comparison with the Raman spectra of various proteins and phospholipids (*5–9*). We confirmed many of these assignments by measuring the Raman spectra of the separated lipid and protein components of the membrane (*12*).

Residue vibrations from most of the predominant amino acids in opsin are shown in Fig. 1 and Table 1. These include vibrations from the following residues: tyrosine (Tyr), 18 residues; phenylalanine (Phe), 30 residues; arginine (Arg), 10 residues; tryptophan (Trp), 10 residues; aspartic acid (Asp), 28 residues; and glutamic acid (Glu), 37 residues (*3*).

Although it has been proposed that opsin contains two disulfide bonds (*3*), no peak is seen in the 500 to 520 cm^{-1} region where the strong S-S vibration usually appears—for example, 507 and 516 cm^{-1} for lysozyme (*19*) and ribonuclease (*20*). It has been predicted (*21*) that the frequency for S-S (ν_{SS}) is 500 to 520 cm^{-1} only if the two C-S bonds are in the common *gauche-gauche* configuration, that ν_{SS} is $\simeq$ 525 cm^{-1} if one C-S is in a *trans* form, and that ν_{SS} is $\simeq$ 540 cm^{-1} if both are *trans*. Since we find weak peaks at both 525 and 540 cm^{-1}, we conclude that either no S-S bonds exist or, if they do exist, they are not in the common *gauche-gauche* configuration.

Table 1. Principal peak frequencies in the Raman spectrum of calf opsin membranes (Fig. 1).

Peak frequency (cm^{-1})	Tentative assignment
327	
375	Protein
418	
460	
487	
525	S-S stretch: *trans-gauche* form
545	S-S stretch: *trans-trans* form, Trp
622	Protein: Phe ring mode
644	Protein: Tyr ring mode
703	
720	Phospholipid: choline
741	Phospholipid
758	Protein
831	Protein: Tyr (hydrogen-bonded OH)
850	Protein: Tyr (free OH)
868	
880	Protein: Trp
930	Phospholipid
974	Phospholipid
1003	Protein: Phe
1033	Protein: Phe
1048	Phospholipid
1068	Phospholipid: C-C stretch of *trans* hydrocarbon chain
1082	Phospholipid, O-P-O and C-C stretch of *gauche* hydrocarbon chain
1127	Protein, phospholipid: C-C stretch of *trans* hydrocarbon chain
1158	Protein, retinal
1175	Protein: Tyr, Phe
1206	Protein: Tyr, Phe
1269	Phospholipid, protein (amide III)
1300	Phospholipid (CH_2), protein (amide III)
1340	Phospholipid, protein: γ (CH_2)
1400	Protein: Asp, Glu ν (COO^-)
1450	Protein, phospholipid: δ (CH_2)
1555	Protein, retinal (-C=C-C=C)
1585	Protein: Phe, Arg
1605	Protein: Tyr, Phe
1618	Protein: Tyr, Phe
1660	Protein (amide I), phospholipid (-C = C-)
1740	Phospholipid: ester carbonyl
2570	Protein: SH stretch

Opsin is also estimated to contain six cysteine (Cys) residues, and we find a weak vibration at 2570 cm^{-1}, which is characteristic of SH groups (*22*).

The intensity ratio, I_{850}/I_{831}, of the 850 to 831 cm^{-1} vibrations (Tyr, doublet) is a sensitive indicator of hydrogen bonding to tyrosine (*23*). For example, in ribonuclease, for which three out of six tyrosine residues are strongly bonded to the COO^- moiety of the aspartic acid residue, $I_{850}/I_{831} = 8/10$ and changes to 10/9 when the aspartic acid residue is protonated (*20*). Furthermore, when ribonuclease is denatured, $I_{850}/I_{831} = 10/6$, indicating even less hydrogen bonding (*20*). In opsin membranes $I_{850}/I_{831} < 1$, indicating that a significant fraction of tyrosine residues is strongly hydrogen bonded. It is possible that a large fraction of the tyrosine residues form hydrogen bonds with aspartic and glutamic acid residues, which constitute 40 percent of the opsin polar residues (*2, 3*).

The amide III vibration (1220 to 1320 cm^{-1}) is an extremely sensitive indicator of the overall skeletal conformation of proteins (*5–9, 16–20*), with an antiparallel-pleated β-sheet between 1229 to 1235 cm^{-1}, a random coil between 1243 to 1253 cm^{-1}, and an α helix between 1265 to 1300 cm^{-1} (*24*). A strong symmetric peak is found at 1269 cm^{-1}, whereas little activity is detected between 1210 and 1250 cm^{-1}. Although part of the 1269 cm^{-1} peak cannot be due to protein [since it is likely that a lipid vibration falls in this region (*12*)], the absence of activity below 1240 cm^{-1} indicates that very little β structure is present. Since deuteration of the opsin during the washing step quickly leads to a 15 percent reduction in I_{1269}/I_{1450} from its value in a control where an H_2O (rather than deuterated) buffer is used, at least part of the 1269 cm^{-1} peak is due to an amide III mode (*8, 9, 18, 25*). In addition, the activity shifts from 1269 to 1250 cm^{-1} upon denaturation of the protein (*12*). This would indicate that part of the native opsin skeletal structure is α-helical, as was predicted on the basis of CD measurements on detergent-extracted rhodopsin (*26*). Recent x-ray measurements on purple membranes (bacteriorhodopsin) (*27*) have also detected α-helical structure. The possibility exists that activity above 1300 cm^{-1} is also due to amide III vibrations [for example, myosin peaks at 1304 and 1320 cm^{-1} have been assigned to amide III α-helical vibrations (*18*)]. Furthermore, deuteration of α helices embedded in a hydrophobic membrane environment might greatly reduce hydrogen-deuterium exchange rates (*28*).

On the basis of previous Raman spectro-

scopic studies of lipids (*8, 9, 29*) and the fact that the 1082 cm^{-1} peak is stronger than the 1068 cm^{-1} peak, we conclude that the opsin membrane phospholipids are in a fluid state at room temperature. This agrees with diffusion studies (*4, 30*). Further, it is likely that the membrane lipids remain fluid down to at least 15°C. Below 15°C the 1068 cm^{-1} *trans* peak becomes more intense relative to the *gauche* peak at 1082 cm^{-1}, an indication of a gel-liquid crystal transition. Such a low transition temperature could be consistent with the high degree of lipid hydrocarbon unsaturation present in opsin membranes (*30, 31*).

In conclusion, Raman spectroscopy of photoreceptor membranes can provide a sensitive probe of the membrane lipids and the membrane protein opsin (*32*). The main picture that has emerged from our study thus far is of an opsin protein which contains α-helical bonding but no detectable β-helical structure or random coil. Thus, the "dumbbell" model (*4*), which envisions significant β-helical structure such as found in gramicidin A (*17*), is unlikely. However, our data are consistent with the three-dimensional model recently obtained for purple membrane, which reveals a protein consisting of seven closely packed α-helical segments perpendicular to the plane of the membrane (*33*). We also find evidence for hydrogen-bonded tyrosines which may be present as Tyr . . . $^{-}$OOC bonds. In addition, the unusual configuration of disulfide bonds could be related to the proposed disulfide exchange during the bleaching process (*34*).

Note added in proof: Recent Fourier transform infrared absorption studies on photoreceptor membranes (*12*) indicate extensive α-helical structure and no detectable β structure for both opsin and rhodopsin.

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Shoot Initiation from Carnation Shoot Apices Frozen to −196°C

Abstract. *Aseptically excised shoot apices from* Dianthus caryophyllus *were frozen to −196°C and subsequently thawed to room temperature. Survival rates as high as 33 percent, determined by callus formation, growth, or chlorophyll formation, are reported; plant formation has been observed.*

During the past few years, several groups have developed techniques that expose plant suspension culture cells (*1*), callus (*2*), and both plant (*3*) and animal (*4*) embryos to temperatures as low as −196°C while maintaining cell viability. The objective of this work has been to obtain a reliable method for preserving specific cell and tissue culture lines for extended periods of time without the risk of microorganism contamination, equipment failure, or changes in chromosomal cytology and morphogenic potential (*5*). These problems are associated with the current practice of serial transfer. Plants have been grown from frozen cells after thawing, but initiation of organized development is difficult to achieve in cell cultures of many species even before freezing. Shoot tip or apex cultures, on the other hand, are routinely used to propagate many species (*6*) and, in addition, give rise to plants that are free of

Fig. 1. Shoot initiation from carnation shoot apices cultured in the light after being frozen to −196°C and thawed under the conditions described in the text. (a) Apex immediately after thawing (1 mm long). (b) Apex 2 months after thawing (7 mm long).